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Molecular biology of calcium channels

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The molecular biology of Ca^{2+} channels has its origins in the biochemical characterization of the skeletal muscle dihydropyridine receptor. These studies established that the dihydropyridine receptor/channel complex was a multi-subunit complex composed of α_1 (the ion-conducting subunit), and smaller accessory subunits (α_2, β, and γ). These subunits were purified, sequenced, cloned, and expressed. Cloning of these cDNAs provided the probes to discover the molecular diversity of Ca^{2+} channels. To date (April 1995), genes for six α_1s, four βs, one α_2, and one γ have been cloned. Preliminary classification schemes divided native calcium channels into low voltage-activated (T-type) and high voltage-activated types: L-type, dihydropyridine-sensitive; and N-type, ω-conotoxin GVIA-sensitive. The development of new toxins has led to the further subclassification of high voltage-activated channels to: P-type, which is blocked by ω-agatoxin-IVa from the funnel-web spider *Agelenopsis aperta*; Q-type, which is blocked by ω-conotoxin-MVIIC from the marine snail *Conus magus*; and R-type, which is resistant to most toxins (DB Wheeler, A Randall, and RW Tsien, *Science* 264:107–111, 1994). Expression studies with cloned α_1s have proven that this subunit determines the voltage and pharmacological sensitivity of the channel. This should allow us to classify the cloned α_1s in terms of their type. Unfortunately these properties are affected by the choice of expression system, and the subunit composition of the channel. Despite these complications, the six α_1s have been classified as follows: three α_1s (α_{1S}, α_{1C}, and α_{1D}) belong to the L-type (dihydropyridine-sensitive); α_{1B} is an N-type; α_{1A} is a P-type although it has also been classified as Q-type; and α_{1E}, which does not display any distinctive pharmacology, has been called an R-type (resistant). We will review the cloning, classification, tissue distribution, and functional expression of these α_1 subunits and the accessory subunits.

Molecular biology of the skeletal muscle L-type α_1, α_{1S}

Biochemistry

Skeletal muscle provided an excellent source for the purification of L-type Ca^{2+} channels since it contains functional channels, and a high density of dihydropyridine (DHP) receptors [1]. Due to the ability of DHPs to affect channel activity, it was assumed that DHP receptors were either on the channel itself or at least closely associated with it. A second important feature of DHPs is that they bind to the receptor/channel with high affinity and dissociate slowly, thereby providing a marker to follow the purification of the receptor. Typically, skeletal muscle t-tubule membranes were prelabeled with tritiated dihydropyridines, solubilized by digitonin or CHAPS, and purified through wheat germ agglutinin chromatography, anion exchange chromatography, and sucrose gradient centrifugation [2–5]. The calcium channel activity of purified DHP receptors was measured after reconstitution into lipid bilayers [6].

Early work revealed the presence of at least three proteins in the purified preparation, termed α, β, and γ. However, in 1987 the existence of two αs became apparent [7–10]. Azidopine photolabeling studies showed that the DHP binding subunit was a 165 to 190 kD protein whose mobility in SDS-PAGE gels was not affected by reducing agents. In contrast, the other protein at ~170 kD, $\alpha_2\delta$, can be dissociated under these conditions, decreasing its mobility to 142 kD (α_2) and 22 to 28 kD (δ). In addition, the purified complex contained a 55 kD β subunit and the 32 kD γ subunit. All these subunits have been purified, sequenced, and cloned [11–15]. The sizes of the purified proteins agree quite well with the sizes deduced from the primary sequence, except α_1, which appears smaller in the purified preparations. At best, trace amounts of the full-length 214 kD α_1 have been detected [16, 17]. This size discrepancy is due to proteolytic processing of the carboxyl terminus either *in vivo* or during the purification process [17]. In addition, the α subunit of G_s copurifies with the DHP receptor [18], supporting the notion that there is a direct pathway between G_s and L-type Ca^{2+} channels.

An important feature of L-type calcium channels is that their activity is stimulated by cAMP-dependent protein kinase (PKA) phosphorylation. Although this regulation may play a more important role in cardiac function [1], a similar cascade operates in skeletal muscle [19, 20]. PKA phosphorylation stimulates the activity of the purified receptor/channel complex [6], and leads to phosphorylation of both the α_1 subunit [21, 22] and the β subunit [23, 24].

Cloning of the skeletal muscle α_1 subunit

Cloning of the skeletal muscle α_1 subunit was accomplished by purifying tryptic peptides derived from the DHP receptor, determining the sequence of these fragments, preparing degenerate oligos based on this sequence, and screening of rabbit skeletal muscle cDNA libraries with these oligos [11]. Although this α_1 cDNA has been called many things (pCAC6 [25], or CaCh1 [26]), it is now called α_{1S}. The full-length cDNA of the rabbit skeletal muscle DHP receptor has an open reading frame that encodes a protein of 212,018 D. Its sequence has 55% similarity to the Na^+ channel α subunit, and like the Na^+ channel, it contains four internal repeats, termed Domains I, II, III, and IV. These structural motifs are found in all the cloned Ca^{2+} channel α_1 subunits. Secondary structure analysis predicts eight membrane

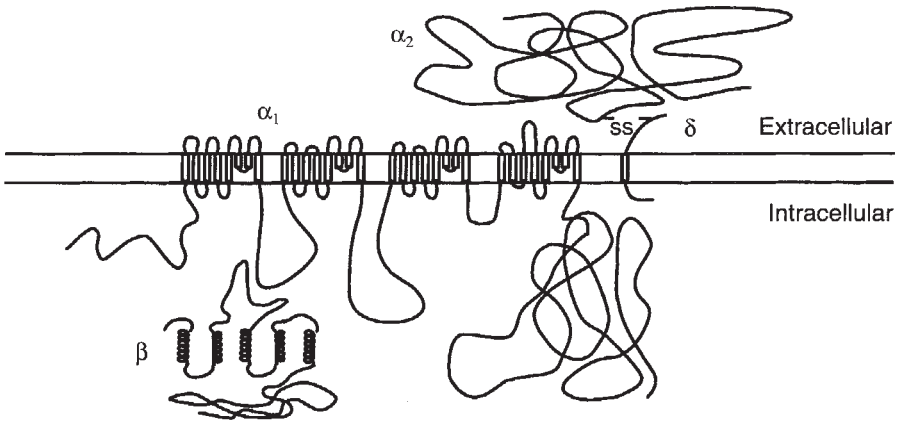

Fig. 1. *Oligomeric structure of voltage-gated Ca^{2+} channels.* This cartoon shows the general structure of a Ca^{2+} channel. The dimensions are only approximate. Membrane spanning (or embedded) regions are shaded. The α_2 subunit is shown to be in the extracellular space according to [121]. The regions where α and β interact is from [139, 140]. The regions where $\alpha_2\delta$ and γ interact is not known. β is shown to contain five helices, the second helix can be deleted by alternative splicing in β_1 and β_2, while it is missing in β_3 and β_4. The secondary structure prediction programs indicate numerous possible helices, but for simplicity only the longest are shown.

spanning regions in each domain of voltage-gated Ca^{2+} and Na^+ channels (Fig. 1, abbreviated IS1, for Domain I, transmembrane segment 1) [27]. This model predicts that the amino and carboxyl termini are located inside the cell, and that there are long cytosolic loops that connect each domain.

Similar α_{1S} cDNAs have been cloned from carp [28], mouse skeletal muscle [29], mouse ovary [26], and mouse BC3H1 cells [26]. The human α_{1S} gene was localized to chromosome 1q31q32 [30]. The mouse skeletal muscle cDNA was cloned from muscular dysgenic (*mdg*) mice. In this mutant there was a single nucleotide deletion that resulted in a frame shift and termination prior to the domain IV S6 loop. Apparently this truncated α_1 is not a functional channel [25].

The α_{1S} gene is alternatively spliced in at least one location, after the IV S3 segment. This splice variation, which appears to be due to exon-skipping, has been detected with both PCR and cDNA library screening [26, 31]. All three L-type Ca^{2+} channel genes are spliced in this same area [26]. The functional significance of this splicing remains a mystery. A second mystery centers on the existence of a two-domain isoform [32], which recent studies have failed to confirm [31].

Northern blot analysis of RNA from different tissues identified a 6.5 kb transcript of α_{1S} in skeletal muscle from rabbit [12, 33], carp [28], mouse [29], and mouse BC3H1 cells [33]. However, its expression appears limited to skeletal muscle since little or no hybridization was detected in heart, brain, trachea, lung, ileum, stomach, or kidney [12, 33]. One exception may be aorta, where it was detected by Koch et al [34], but not by Ellis et al [12].

Functional expression

A popular system for expressing cloned ion channels is the *Xenopus laevis* oocyte [35]. Unfortunately, microinjection of α_{1S} cRNA did not lead to the expression of functional Ca^{2+} channels (measured with Ba^{2+}), even when injected with auxiliary subunits (Perez-Reyes, unpublished observations). Therefore, we chose stable transfection of mouse L cells as an expression system [36]. These studies proved that expression of α_{1S} without any other subunits led to the induction of functional Ca^{2+} channels. Surprisingly the activation kinetics of these currents was extremely slow, suggesting a role for the other subunits. To test this hypothesis we coexpressed the skeletal muscle β with α_{1S} in L cells and indeed, coexpression of β with α_1 led to a normalization of the current kinetics [37]. This coexpression also led to fivefold increases in high-affinity DHP binding sites. Paradoxically there was not a corresponding increase in the current density. Anyway, these results showed that β was a subunit, which could alter the biophysical properties of the channel and alter the expression of DHP binding sites.

An alternate expression system used was microinjection of an expression plasmid into cultured dysgenic *mdg* myotubes, which restored both Ca^{2+} current and skeletal muscle-like excitation-contraction coupling, contraction that occurs even in the absence of external Ca^{2+} [25].

Molecular biology of the cardiac muscle L-type α_1, α_{1C}

Biochemistry

Purification of cardiac Ca^{2+} channels has been hampered by a low density of DHP binding sites in sarcolemmal membranes from heart [38]. Despite this limitation, the channel has been purified from various species. These studies have identified at least three subunits of the L-type Ca^{2+} channel: (1) an α_1, whose apparent molecular weight is between 165 and 195, (2) an $\alpha_2\delta$, and (3) a β [39–45].

In contrast to skeletal muscle preparations, PKA phosphorylation of purified cardiac α_1 was not observed in purified heart preparations [42, 43, 46, 47]. The size of the purified α_1 is much smaller than the size deduced from the cDNA clone, suggesting proteolysis. Similar to the skeletal muscle α_{1S} this truncation occurs at the carboxyl terminus, since antibodies directed against this region only recognize a minor full-length form (242-kDa) in heart and brain preparations [45, 48]. Yoshida et al [48] showed that only this long form could be phosphorylated by PKA.

The purified cardiac DHP receptor also contains a 170 kD $\alpha_2\delta$ subunit, which after reduction is split into a 140 kD α_2 and a 30 kD δ [42–44]. This $\alpha_2\delta$ is either identical to the skeletal muscle form, or a splice variant.

The cardiac β was purified using a specific anti-peptide antibody directed against the β_2 sequence [47]. This antibody immunoprecipitated DHP-labeled receptors from a solubilized canine cardiac preparation, thereby proving that β_2 is the predominant β in heart. Back-phosphorylation studies showed that *in vivo* isoproterenol treatment led to the phosphorylation of β_2 [47].

Cloning of the cardiac α_1

The α_{1C} (CaCh 2, CARD, or rbC) cDNA was cloned from rabbit heart cDNA libraries using low stringency hybridization

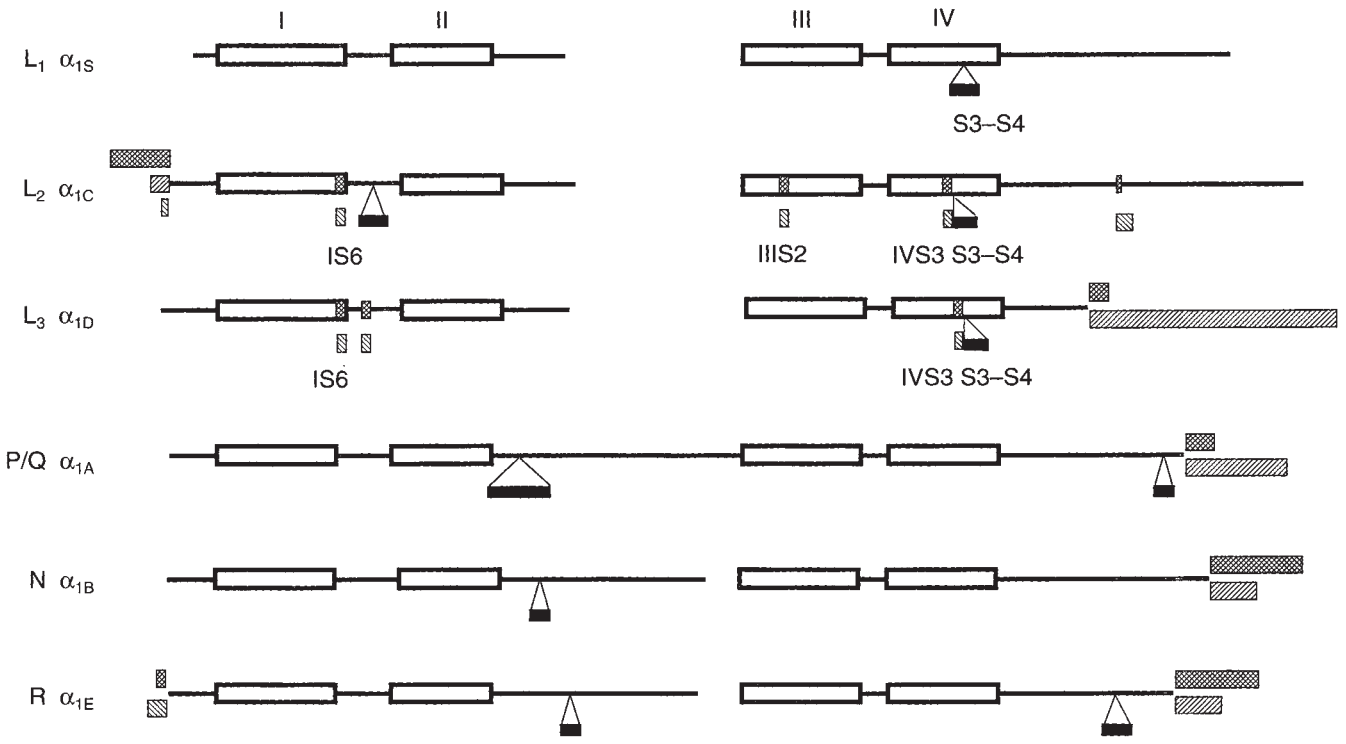

Fig. 2. *Alternative splicing of α_1 subunits.* The regions where alternative splicing of exons occurs is shown by the hatched boxes. Insertion/deletions (of > 10 amino acids) are shown by the thick line. The diagram is roughly to scale.

with skeletal muscle α_1 cDNA as probe [49]. Its full-length cDNA contains an open reading frame that encodes a protein of 242,771 D. This protein is 66% similar to α_{1S}. The human cardiac α_{1C} has also been cloned and its gene mapped to chromosome 12p13 [50].

Northern analysis detects α_{1C} expression in heart, brain, aorta, trachea, and lung [33, 49, 50]. Expression of α_{1C} has also been detected in the following tissues and cell lines: kidney [51], mouse ovary [26], pancreas [52], colonic smooth muscle [53], fibroblasts [54], HIT cells [26], GH_3 cells [55], PC12 cells [55], NE-115 cells [55], and AT-20 cells [55]. In addition, α_{1C} is transiently expressed in skeletal muscle during development [56].

Comparison of the published sequences reveals that this gene is alternatively spliced in at least six regions (Fig. 2). Due to the inherent difficulties in isolating an 8.5 kb cDNA, it has been difficult to decide if there are truly tissue specific forms of α_{1C}. In other words, are all six splice regions coordinately regulated, or are there really 64 differently spliced α_{1C}s? At least one region appears to be spliced in a tissue specific manner, which is the amino terminus, where there is a cardiac specific form while the shorter variant is expressed in smooth muscle and brain [33]. The relative abundance of the two alternatively spliced IV S3 segments has been found to vary between brain regions [57] and development [58, 59]. Although the functional significance of each of these splice sites has not been determined, a difference was noted between the cardiac and lung versions (3 alternatively spliced regions) in terms of their sensitivity to the DHP antagonist, nisoldipine [60]. These results suggest that splicing of α_{1C} may contribute to the selectivity of DHP antagonists for smooth muscle over cardiac L-type Ca^{2+} channels. However, the only full-length vascular smooth muscle α_{1C} cloned [61] does not contain the splice variant regions found in the lung clone. Other strange splicing events, which are not predicted to encode functional channels, have also been reported [54, 62].

Functional expression

Injection of α_{1C} cRNA into *Xenopus* oocytes leads to the appearance of dihydropyridine-sensitive Ba^{2+} currents [49, 50, 63, 64]. In contrast to the stable transfection systems required to express α_{1S}, oocytes provided an easy assay system to test the effects of auxiliary subunits. Mikami et al [49] showed that $\alpha_2\delta$ could increase α_{1C} currents approximately twofold, with no apparent changes in the properties of the channel. Similar results were reported by Biel et al using the lung variant of α_{1C} [65]. Bolstered by our experience with the skeletal muscle β, we recloned the cardiac α_{1C} and coexpressed it with β [63]. We found that β not only caused an increase in the amplitude of the currents, but also shifted the voltage-dependence of activation and accelerated the rate of activation. We have also found that all βs modulate α_{1C} activity similarly. These effects can be classified as either quantitative, βs increase the amount of functional channels, or qualitative, βs affect the biophysical properties of the channel.

Coexpression of γ with α_{1C} has little or no effect on the currents in oocytes, although coexpression of γ with $\alpha_{1C}\beta_{1a}$ led to a small increase in the currents [63]. In contrast, Singer et al [64] reported that γ had profound effects on inactivation. Many results of this paper [64] have been difficult to reproduce, and their currents were likely contaminated with endogenous oocyte channels [66, 67]. Interestingly, these endogenous channels are regulated by mammalian subunits [64, 68]. For example, the β subunit alone

Table 1. Cloning and expression of cloned calcium channel α_1 subunits

Type	α_1	Source	Accession#	Expression	Pharmacology
L_1	α_{1S}	Rabbit skeletal muscle [11, 12]	M23919, X05921	L cells [36]	Dihydropyridines, verapamil, diltiazem, [152]
		Carp [28]	M37203		
		Mouse *(mdg)* [29]	L06234		
L_2	α_{1C}	Rabbit heart [29]	X15539	Oocytes [49], CHO [60], COS [132], HEK [74], Sf9 cells [72]	Dihydropyridines
		Rabbit lung [65]	X55763	Oocytes [65], CHO cells [60]	Dihydropyridines
		Rat aorta [61]	M34364	Oocytes [153]	
		Mouse brain [154]	L01776	Chromaffin cells [154]	
		Rat brain [57]	M67515, 6	Oocytes [155]	Dihydropyridines
		Human fibroblasts [54]	M92269		
		Human heart [50]	L04569	Oocytes [50]	Dihydropyridines
L_3	α_{1D}	Hamster HIT [80]			
		Human pancreas [79]	M83566		
		Human brain [68]	M76558	Oocytes [68]	Dihydropyridines
		Rat brain [78]	M57682		
P/Q	α_{1A}	Rabbit brain [84]	X57476, 7 X57688, 9	Oocytes [84, 90]	ω-CTx-MVIIC, ω-agaIVA
		Rat brain [85]	M64373	Oocytes [86]	ω-CTx-MVIIC, ω-agaIVA
N	α_{1B}	Ray [111]	L12532		
		Rat brain [103]	M92905	Oocytes [107]	ω-CTx-GVIA
		Rabbit brain [104]	D14157	Myotubes [104]	ω-CTx-GVIA
		Human brain [102]	M94172, 3	HEK293 cells [108]	ω-CTx-GVIA
		Mouse brain [105]	U04999		
R	α_{1E}	Ray [111]	L12531	Oocytes [114]	Resistant
		Rat brain [110]	L15453	Oocytes [110]	
		Rabbit brain [109]	X67855 X67856		
		Human brain [112, 113]	L27745 L29384 L29385	Oocytes [112] and HEK-293 cells [113]	Resistant
		Mouse brain [113]	L29346		

can increase these currents up to 500 nA [69]. This contamination by endogenous currents casts into doubt many studies where α_{1C} expression was low (< 100 nA) [70, 71].

Other systems used to express α_{1C} include: baculovirus infection of the insect cell Sf9 [72, 73], COS cells [74], CHO cells [48, 75], L cells [76], and HEK-293 cells [74]. These studies have verified the essential role of β in increasing the functional expression of DHP binding sites and channel activity [76, 77].

Molecular biology of a neuroendocrine L-type α_1, α_{1D}

Cloning of α_{1D}

Three approaches were used to clone a third L-type α_1 (CaCh 3, rbD): (1) low stringency hybridization using α_{1S} cDNA as probe [68, 78], (2) PCR [26, 79], and (3) oligonucleotides based on conserved regions between α_{1S} and α_{1C} [80]. The human α_{1D} was cloned from pancreatic islet cDNA libraries [79]. The human gene was localized to chromosome 3 [81].

The deduced amino acid sequence is very similar to α_{1C}. Even the location and sequence of alternatively spliced regions are similar (Fig. 2). In addition, novel splice variants of the carboxyl terminus have been cloned. One of these versions is considerably shorter than any of the other L-type α_1s (whose C termini make up to 30% of the protein). Two size forms of α_{1D} have also been detected in immunoprecipitates from brain preparations [45].

Analysis of the distribution of α_{1D} using Northern blots has been complicated by the cross-reactivity of the three L-type α_1s. For example, in one study the α_{1D} probe cross-reacted with a 6.5 kb mRNA in skeletal muscle and aorta (presumably α_{1S}) [78]. A second confounding factor is that α_{1C} and α_{1D} have similarly sized mRNAs (8.5 vs. 8.6, respectively). In a second study using the 5′ untranslated region, which bears no sequence homology to α_{1C} (although it is surprisingly conserved across species, [82]), detected expression in brain and pancreas, with traces observed in cardiac and skeletal muscle [80]. The cross-reacting species detected in heart was slightly smaller than the mRNA in brain and pancreas.

Despite these complications, α_{1D} is clearly expressed in the following tissues and cell lines: pancreas [79, 80], brain [68, 78], kidney [51], ovary [26], GH_3 cells [55], PC12 cells [55], NE-115 cells [55], and AT-20 cells [55]. The distribution in brain was mapped by PCR [68, 80], *in situ* hybridization with cDNA probes [83], and immunofluorescence with antibody probes [45].

Functional expression

Despite having been cloned by so many groups, only the human brain α_{1D} has been expressed [68]. Injection of α_{1D} cRNA into oocytes did not lead to the appearance of detectable currents. However, coinjection of $\alpha_2\delta$ and β with α_{1D} did lead to the appearance of DHP-sensitive currents. Surprisingly, injection of $\alpha_2\delta$ and β without α_{1D} also led to the appearance of currents, however, these currents were not DHP-sensitive. This result suggests that mammalian subunits can boost the expression of endogenous oocyte channels. A second surprising result was that the $\alpha_{1D}\alpha_2\delta\beta$-induced currents were partially blocked by a high concentration of ω-conotoxin (Ctx) GVIA [68]. Although this result may be expected on the sequence similarities between L

Table 2. Cloning and expression of calcium channel α_2, β, and γ subunits

Subunit	Splice variant	Source	Acession#	Tissue distribution	Expression with α_1
$\alpha_2\sigma$	α_{2a}	Rabbit skeletal muscle [12]	M21948	Skeletal muscle	Increases α_1 currents in occytes [49], increases binding [118]
	α_{2b}	Rat [123]	M86621	Brain	
		Human brain [68]	M76559	Brain	Increases binding and currents [108]
	α_{2c}, α_{2d}, α_{2e}	Human aorta [108]		Aorta	
β_1	β_{1a}	Rabbit [13]	M25817	Skeletal muscle	Increases currents, shifts IV [63], alters kinetics [37], increases binding [37]
		Mouse (unpublished)	M25514	Skeletal muscle	
		Human skeletal muscle [130]	M92301	Skeletal muscle	
		Human heart [129]	L06112	Heart	
	β_{1b}	Rat [156]	X61394	Brain	
		Human brain [130, 108]	M92303	Brain	Increases binding [108]
		Human heart [129]	L06110	Heart	
	β_{1c}	Human brain [68, 130]	M76560 M92302	Brain, heart, spleen	Increases binding [108]
		Human heart [129]	L06111	Heart	
β_2	β_{2a}	Rat brain [132]	M80545	Brain, heart, lung	Increase currents, shift IV [132]
		Rabbit heart [71]	X64297	Brain, heart, aorta	Increase currents [71]
	β_{2b}	Rabbit heart [71]	X64298	Brain, heart, aorta	Increase currents [71]
	β_{2c}	Rabbit brain [71]	X64299	Brain	
	β_{2c}	Human brain [133]		Brain	
β_3		Rat brain [134]	M88751	Brain, lung	Increases currents, shifts IV, affects inactivation [134]
		Rabbit heart [71]	X64300	Brain, aorta, trachea, lung	Increase currents [71]
		Human brain [135]	L27584 X76555 U07139	Brain, ovary, and colon	Increases currents [135]
β_4		Rat brain [136]	L02315	Brain, kidney	Increases currents, shifts IV, affects inactivation [136]
γ		Rabbit skeletal muscle [14, 15]	M32231	Skeletal muscle, lung	Little or no effect on currents [63]
		Human skeletal muscle [125]	L07738	Skeletal muscle	

and N-type channels, an alternate explanation is that ω-CTx-GVIA was blocking the endogenous oocyte channel [69]. Apparently oocytes can express two Ca^{2+} channels, an N-type and a T-like channel [69].

Molecular biology of a brain α_1, α_{1A}

Cloning of α_{1A}

The α_{1A} cDNA (CaCh 4, BI, or rbA) was cloned using low stringency hybridization of rabbit and rat brain cDNA libraries using the skeletal muscle α_{1S} cDNA as probe [84, 85]. Its sequence is approximately 40% identical to the L-type α_1s. In general the membrane spanning regions are well conserved, while the connecting loops are not.

Splice variation occurs in three regions (Fig. 2). The two carboxyl terminus variants were termed BI-1 and BI-2. The rat and rabbit sequences differ in seven regions within the long II-III loop; one region is due to alternate splicing of exons while the other six are insertion/deletions. In PCR of kidney cDNA, a small deletion was found in the same region (IV S3-S4 loop) as described for the L-type α_1s [26, 51].

Northern blot analysis indicates that α_{1A} is predominantly expressed in brain, with much less detected in heart, and none in skeletal muscle, stomach, or whole kidney [84]. Northern analysis of different brain regions showed high expression in cerebellum, but it was also found in hippocampus, olfactory bulb, and spinal cord [85]. *In situ* localization studies detected α_{1A} RNA throughtout the rat brain with higher levels found in the cerebellum, cerebral cortex, and hippocampus [86]. By using mice with different types of cerebellar degeneration, it was found missing in mice with Purkinje cell degeneration [84]. Its expression has also been detected in kidney cortex, where it is expressed in the distal convoluted tubule [51].

Functional expression

Injection of α_{1A} cRNA into oocytes led to the appearance of small Ba^{2+} currents [84]. Coexpression with γ had no effect, while currents were increased by either $\alpha_2\delta$ (3-fold), or β_1 (20-fold) [84]. Coexpression with both $\alpha_2\delta$ and β_1 stimulated the currents over 200-fold to 6.5 μA, showing a synergistic action. Single channel analysis showed that this channel had a conductance of 16.5 pS in 110 mM Ba^{2+} [84]. Coexpression with β_{1b} or β_3 shifted the current-voltage curve to more negative potentials and accelerated inactivation kinetics [86]. Surprisingly, β_{2a} slowed inactivation kinetics [86]. This slowing of inactivation kinetics has also been observed on the endogenous T- and N-type oocyte channels [69], as well as with α_{1E} [87].

The pharmacology of the α_{1A}-induced currents has presented a quandary in the classification of this channel, leading to its classification as both P-type (ω-agatoxin IVA sensitive) and Q-type (ω-CTx MVIIC sensitive) [88]. It was insensitive to DHPs and ω-CTx GVIA, but could be blocked by crude venom from the funnel-web spider, *Agelenopsis aperta* [84]. This result, along with its distribution in cerebellar Purkinge neurons [89], suggested that α_{1A} represented a P-type channel. However, the crude venom

contains many toxins capable of blocking high-voltage activated Ca^{2+} channels. The expressed channel was blocked by ω-agatoxin-IVA, which is selective for P-type channels [86, 90, 91]. However, P-type channels *in vivo* are ~100-fold more sensitive to ω-agatoxin-IVA, and 10-fold less sensitive to ω-CTx MVIIC, than α_{1A}-induced currents in oocytes [90]. These differences led to the suggestion that α_{1A} represents a new type of channel, termed Q-type [88]. However, these pharmacological differences may be the result of other differences, such as either improper post-translational modifications, interference from endogenous oocyte channels, splice variations, or subunit coexpression. Similar discrepancies between native cardiac L-type channels and cloned α_{1C} channels were noted [90]. For α_{1C}, both splice variations [60] and subunit coexpression [37] have been shown to affect the channel's pharmacology.

Molecular biology of an N-type channel, α_{1B}

Biochemistry

The N-type Ca^{2+} channel was purified from brain using ω-CTx-GVIA as a marker [93, 94]. The purified channel has also been reincorporated into lipid bilayers, where it displayed functional activity [95]. Similar to L-type Ca^{2+} channels, the purified complex contains an α_1 (α_{1B}), $\alpha_2\delta$, and a β (β_3) [94]. Other proteins that have been purified with the ω-CTx-GVIA receptor are: a 95-kDa protein [94], syntaxin and synaptotagmin [96], and the α subunit of G_o [97]. Apparently Ca^{2+} channels can be bimodally regulated by direct interaction with G proteins; they are inhibited by G_o [98, 99] and stimulated by G_s [100].

Antibodies, raised against peptides from the deduced amino acid sequence of either the β subunit or α_{1B}, can immunoprecipitate ω-CTx-GVIA receptors [45, 101]. The α_{1B} specific antibodies recognized two size forms that differed in the carboxyl terminus [45]. Similar to the L-type α_1s, these two isoforms were also differentially phosphorylated, in this case by CaM kinase II.

Cloning of α_{1B}

The α_{1B} cDNA (CaCh 5, BIII, rbB) was cloned by low stringency screening of brain cDNA libraries using the skeletal muscle α_{1S} cDNA as probe [102, 103]. It has also been cloned using α_{1A} cDNA as probe [104] or using PCR with primers against conserved domains [51, 105]. A similar cDNA has also been cloned from the marine ray *Discopyge ommata* [106]. The deduced amino acid sequence of α_{1B} is very similar (82%) to the α_{1A} sequence, but less similar to the L-type α_1s (43 to 51%); a full matrix comparing these sequences is published in [104].

Two variants of the carboxyl terminus were reported for the human α_{1B} [102]. Deletion/insertions of 22 amino acids have also been reported for the II-III loop [105]. The mouse gene has been mapped to chromosome 2 [105].

Northern blot analysis identified a 9.3/9.5 kb doublet in rabbit brain [104]. No cross-reacting species was detected in either skeletal muscle, heart, or stomach. PCR analysis has confirmed this lack of expression in peripheral tissues, including testis, kidney, spleen, adrenal, liver, lung, and pituitary [103]. *In situ* hybridization to mouse brain slices indicates that it has a widespread, but non-uniform, distribution [105]. However, different distributions within the brain have been noted between species [103, 104].

Functional expression

Many systems have been used to express α_{1B}. Williams et al [102] used transient transfection of HEK-293 cells, Fujita et al [104] used dysgenic myotubes, and Stea et al used oocytes [107]. In all these studies the resulting currents were sensitive to ω-CTx-GVIA. The single channel conductance was estimated to be 15 pS using 110 mM Ba^{2+} as the charge carrier [104].

Coexpression of $\alpha_2\delta$ with α_{1B} had little or no effect on currents in oocytes, while β_{1b} led to a fourfold increase in the currents, and an acceleration of activation and inactivation kinetics [107]. Coexpression of $\alpha_{1B}\alpha_2\delta\beta_{1b}$ led to even larger currents with no change in other parameters. Similarly, in HEK-293 cells, coexpression of all three subunits (using stoichiometric ratios of plasmid designed to improve the ratio of mRNA, and hopefully protein) led to the largest currents and to the most ω-CTx-GVIA receptors [108]. Although this complex led to more receptors, the highest affinity receptor was formed by $\alpha_{1B}\beta_{1b}$ complexes. Again, this provides evidence that subunit composition can lead to differences in the pharmacological behavior of the channel.

Molecular biology of an R-type channel, α_{1E}

Cloning of α_{1E}

The α_{1E} cDNA (CaCh 6, BII, rbE) was cloned by either screening cDNA libraries with α_{1A} cDNA as probe [109], or using PCR with primers based on conserved sequences [110–112]. A similar cDNA has also been cloned from rat brain [110], mouse brain [113], human brain [112, 113], and marine ray [114].

Again, splice variants of the carboxyl terminus have been cloned [109]. Insertion/deletion events have also been found in the II-III loop. This loop also contains the repeated motif R-R-H-R-R/H-R. In addition, this loop contains many consensus phosphorylation sites. Another potentially interesting sequence motif in α_{1E} is the presence of two putative Ca^{2+} binding regions [112].

Northern blot analysis identified at least two transcript sizes in rabbit brain [109]. This gene is expressed exclusively in the CNS, since no signal was detected in skeletal muscle, heart, stomach, or kidney RNA. Its distribution in brain was mapped by both Northern analyses [109] and *in situ* hybridization [110]. Significant expression occurs in the olfactory bulb, cortex, hippocampus, and cerebellum.

Functional expression

Robust (1 μA) Ba^{2+} currents were recorded from oocytes injected with α_{1E} alone [112]. Coexpression with β_{1b} had no apparent effect on peak currents, but did shift activation and inactivation to more negative potentials [110]. These currents activate and inactivate relatively quickly [114]. Comparison of the voltage dependence of activation using similar ionic conditions (40 mM Ba^{2+}) shows that α_{1E} currents activate 15 mV more negatively than α_{1C} currents [110]. This led to the suggestion that α_{1E} represented a member of the low voltage-activated family (T-type). However, T-type channels activate 50 mV more negative than L-type channels [115]. A second major difference is that T-type channels have a conductance of ~8 pS, while α_{1E} currents have a conductance of 14 pS [112].

The pharmacology of α_{1E}-induced currents is notable in that most Ca^{2+} channel blockers have no effect. These currents were not affected by DHPs, phenylalkylamines, conotoxins, or FTX

[110, 112–114]. They are partially blocked by crude spider venom, amiloride, Ni^{2+}, and Cd^{2+}. Similarly unresponsive high-threshold Ca^{2+} currents have been described in neurons [116], [92]. These resistant currents have been attributed to a new type of channel called R-type. Since these currents share kinetic and pharmacological characteristics, it has been suggested that α_{1E} represents an R-type channel [92].

Molecular biology of α_2

The α_2 subunit is a large glycoprotein that binds well to wheat-germ agglutinin affinity columns. It was this property, and the ability of CHAPS or digitonin to solubilize the Ca^{2+} channel as a complex, that allowed purification of skeletal muscle α_1. As discussed earlier, α_2 was originally believed to be the DHP receptor. Thus, it was purified and microsequenced [12, 117, 118].

Complementary DNA libraries were screened with degenerate oligonucleotides whose sequence was based on the microsequenced protein. The full-length rabbit skeletal muscle cDNA contains an open reading frame that encodes a protein of 125,018 D [12]. The human $\alpha_2\delta$ gene has been localized to the proximal long arm of chromosome 7q [119].

The δ peptide, which is bound to α_2 through disulfide bonds, was also purified and sequenced [120, 121]. Comparison of this sequence to the cloned α_2 proved that these proteins were encoded on the same cDNA. Apparently $\alpha_2\delta$ is synthesized as a propeptide that gets cleaved near the carboxyl terminus to form δ, however, they remain covalently linked through a disulfide bond. Secondary structure prediction programs suggest that δ contains a transmembrane segment. This may serve to anchor the $\alpha_2\delta$ complex to the membrane (Fig. 1), since α_2 can be extracted with a high pH wash in the presence of reducing agents [121].

Northern blot analysis detects 7 and 8 kb transcripts for $\alpha_2\delta$ mRNA. These mRNAs are expressed in skeletal muscle, heart, aorta, lung, ileum, and brain [12, 33]. A cross-reactive species was also detected in *Xenopus* oocyte RNA [122]. Its distribution has also been mapped by *in situ* hybridization of whole rat embryo [123].

The human and rat brain $\alpha_2\delta$ cDNAs have also been cloned [68, 123]. While most of the amino acid differences between the rabbit, rat, and human sequence can be ascribed to species differences, one region appears to be alternatively spliced. PCR was used to amplify a cDNA that encoded this region, and oligonucleotides specific for the inserted sequence were used to screen Southern blots of the PCR reaction. This study [68], and a second study using rat tissues [123], showed that the first inserted sequence, referred to as α_{2a}, was only expressed in skeletal muscle, while the deleted version, α_{2b}, was expressed in aorta and numerous brain regions. In addition, PCR of human aorta has identified α_2 variants that lack both of these small exons (α_{2c} and α_{2d}), as well as identifying a third exon that that is occasionally skipped [108]. The functional significance of these variations is unknown.

The expression of $\alpha_2\delta$ with α_1s appears to enhance the expression of α_1, especially in the presence of the other accessory subunits, such as β. This suggests that $\alpha_2\delta$ may play a role in determining the spatial distribution of Ca^{2+} channels. It appears that all Ca^{2+} channel types contain $\alpha_2\delta$, although this has only been proven biochemically for the brain N-type [94] and the L-type channels in skeletal and cardiac muscles. Coexpression of $\alpha_{2a}\delta$ with α_{1C} in CHO cells led to an acceleration of inactivation kinetics [124]. In contrast, coexpression of $\alpha_{2a}\delta$ with α_{1B} does not affect kinetics [108].

Molecular biology of γ

The γ protein was purified from skeletal muscle as a complex with the DHP receptor. The protein was sequenced, oligonucleotides were made using this sequence information, and its cDNA was cloned from skeletal muscle cDNA libraries [15]. It was also cloned using antibodies against γ to screen a λgt-11 expression library [14]. The deduced amino acid sequence encodes a protein of 25 kD, which contains four putative membrane spanning regions.

The human γ has been cloned from both cDNA and genomic DNA [125, 126]. The human gene was localized to chromosome 17q24.

Northern blots show that γ is almost exclusively expressed in skeletal muscle [14, 15], although similarly sized transcripts were detected in lung [14] and aorta [33]. PCR results have confirmed this restricted tissue distribution [125].

Expression studies have not determined what role γ plays in the Ca^{2+} channel complex. Its almost exclusive expression in skeletal muscle suggests that it may have a role in excitation-contraction coupling.

Molecular biology of β_1

Biochemistry

Skeletal muscle β was purified with the DHP receptor. The ability of antibodies prepared against this 55 kD protein to precipitate bound DHPs proved that it was part of the receptor/channel complex [127]. *In vitro* treatment of purified DHP receptor preparations with PKA or PKC leads to the phosphorylation of both α_{1S} and β [128]. The phosphorylation of β occurs on at least two residues: Ser-182 [13] and Thr-205 [16].

Cloning of β_1

The β_1 cDNA was cloned using oligonucleotides based on the protein sequence of a fragment of the 55 kD β [13]. Its cDNA contained a 1.8 kb open reading frame encoding a protein of 58 kD. Secondary structure analysis predicts that the protein contains many α helices. These helices contain many charged amino acid residues, indicating that they are probably not embedded in the lipid membrane. The deduced amino acid sequence also contains interesting motifs such as heptad repeats, PEST sequences, and consensus sequences for many protein kinases [13].

The β_1 gene is alternatively spliced in two regions to form at least three full-length proteins (a fourth splice variant, β_{1tr}, appears to result from a splice error that causes a jump in the open reading frame and truncation). One insertion/deletion region occurs in the middle of the sequence (Fig. 3); the brain variants (β_{1b} and β_{1c}) lack an exon used in skeletal muscle β_{1a} that encodes 52 amino acids. The second region is the carboxyl terminus, where alternate exons encode a short (β_{1a} and β_{1c}) or a long tail (β_{1b}). Similarly spliced forms have been detected in human heart and brain [68, 129]. The human gene has also been cloned [130]. The genomic structure confirms that these alternate forms are derived from splicing of one gene. The human gene was localized to chromosome 17 [131].

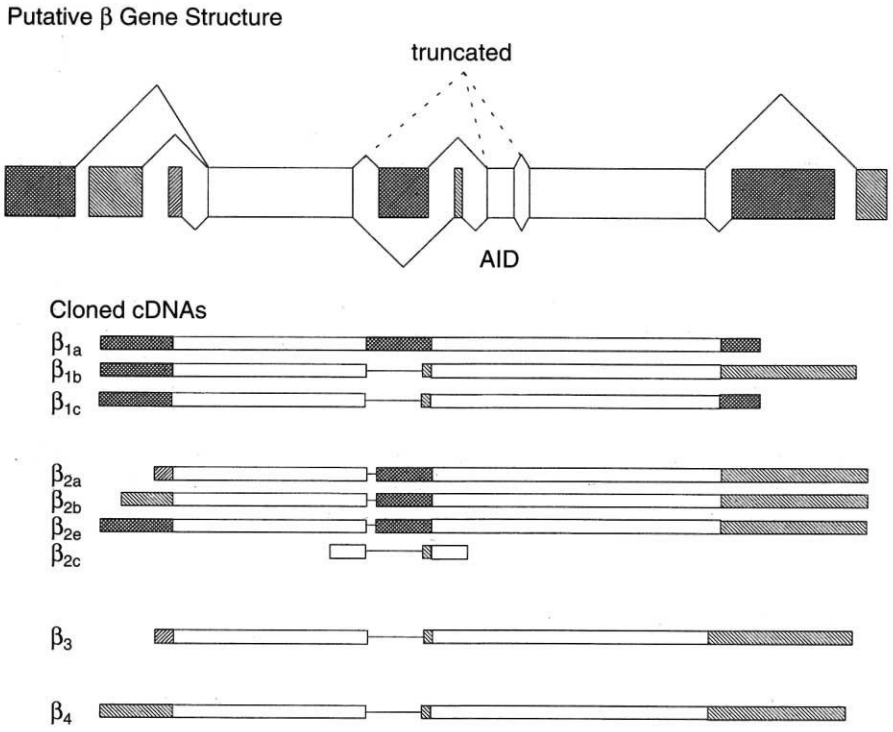

Fig. 3. *Alternative splicing of β subunits from the cloned splice variations.* We have constructed a simplified diagram showing a model of the consensus gene. Exons showing sequence identity are marked in a similar manner. The diagram is roughly to scale. The exons that are skipped in the trunctated forms are shown by dotted lines. The exon that contains the α_1 interaction domain is labeled AID.

Functional expression

In contrast to $\alpha_2\delta$ and γ, coexpression of β with α_1 leads to profound differences in the expression of functional channels and in their biophysical behavior. These studies on skeletal muscle α_1 showed that β could affect activation kinetics and increase the number of DHP binding sites, however, there was no change in current density [37]. Other coexpression studies have used heterologous expression of β_1 with other α subunit types.

Coexpression of β_1 with cardiac α_{1C} also leads to increases in the number of DHP binding sites [132], and as one would predict, it increases the expressed current [63]. In oocytes, we found that β_1 increased the peak currents, accelerated activation, and shifted the voltage dependence of activation to hyperpolarizing potentials [63].

Coexpression of β_1 with N-type α_{1B} in oocytes also led to increases in the currents and altered their kinetics [107]. However, no effect was observed on the voltage dependence of activation. Studies expressing α_{1B} in HEK-293 cells have shown that β_1 increases the number of ω-CTx-GVIA receptors [108].

Coexpression of β_1 with brain α_{1A} in oocytes also led to an increase in the measured currents [84]. However, β did not affect the kinetics of this current. These results show that β_1 can interact with many types of α_1. The most obvious affect of this interaction is to increase the currents measured.

Molecular biology of β_2

Cloning of β_2

During our screening of rat brain cDNA libraries with β_{1a} we found two types of clones: one type was the splice variant, β_{1b}, while the second appeared to be encoded by a distinct gene [132]. Therefore, we called it β_2. Using similar methods β_2 was cloned from rabbit heart [71]. A human brain β_2 cDNA was cloned using sera from Lambert-Eaton myasthenic syndrome (LEMS) patients to screen an expression library [133]. Note, there was an apparent sequencing error of the human β_{2e} form (deletion of cytosine 1603), which resulted in a jump in the reading frame and an apparent lack of homology at the carboxyl terminus [133]. However, addition of the missing nucleotide restores an open reading frame that is nearly identical to the rat and rabbit β_2s.

The β_2 gene is alternatively spliced in at least two regions (Fig. 3). Two versions of the amino terminus were cloned from rabbit heart: one form was identical to the rat brain so it was called β_{2a}, while the other form had a unique amino terminal sequence suggesting the use of an alternate exon (β_{2b}). The human brain β_2 also contains a unique amino terminal sequence, which we call β_{2e}. The second region of alternate splicing occurs in the central region. Apparently all four β genes have intron/exon junctions in this region (Fig. 3). In the β_2 gene there appears to be three exons that are alternatively spliced in this region. PCR cloning has detected versions where none of these three exons is used, however, this results in a frame shift and truncation, β_{2tr}. In contrast to β_1, alternate splicing of the carboxyl terminus has not been detected.

Northern blot analysis detects abundant β_2 transcripts in brain, heart, and lung [132]. Lower levels of expression can also be detected in kidney. At least three transcript sizes were detected (6, 4, and 3.5 kb). This size heterogeneity is probably due to both alternatively spliced mRNAs and alternate use of polyadenylation signals [132].

Functional expression

Coexpression of β_2 with α_{1C} has profound effects on the size and kinetics of the induced currents. Peak currents are stimulated over tenfold. This stimulation is largest at negative test potentials,

which is due to β_2's ability to shift the activation threshold of the channel approximately −10 mV [132]. β_2 also accelerated the activation kinetics of α_1.

Inactivation kinetics of α_{1C} in oocytes are also affected by β_2. Note that the kinetics of α_{1C} in oocytes is much slower than observed when α_{1C} is expressed in other systems. This is especially true of inactivation; these currents in oocytes hardly inactivate below +20 mV test potentials (and above +20 mV there are contaminating currents), while $\alpha_{1C}\beta_2$-induced currents partially inactivate (600 ms τ).

Molecular biology of β_3

Cloning of β_3

Three approaches were used to clone β_3: (1) Hullin et al [71] used the skeletal muscle β_{1a} cDNA as probe, (2) we used a PCR-based strategy using primers that matched conserved regions in β_1 and β_2 [134], and (3) Witcher et al [94] used antibodies raised against the purified N-type channel β protein to screen a λgt11 expression library. The human β_3 was recently cloned and mapped to chromosome 12q13 [135]. The deduced amino acid sequence of the rat β_3 is 98% identical to the human β_3 sequence, while it is only ~70% identical with the other β proteins. Most of this similarity is in a central core region (Fig. 3), while the carboxyl termini are more divergent. The β_3 protein does not contain consensus PKA phosphorylation sequences.

In contrast to β_1 and β_2, alternative splicing of β_3 has not been discovered. It expresses a small exon in the central splice site that has a similar sequence as β_{1b}, β_{2c}, and β_4 (consensus sequence AKQKQKX). Although this exon can be skipped, the reading frame is lost resulting in a truncated protein (β_{3tr}).

Northern blots show that β_3 is predominantly expressed in brain, with lower amounts detected in aorta, trachea, lung, and the pancreatic β-cell lines HIT and RIN [134], [71]. In human tissues it is found in brain, ovary, and colon [135]. Despite having been cloned from heart, little or no mRNA is detected on Northern blots.

Functional expression

The effects of β_3 have been tested on α_{1C}- and α_{1A}-induced currents in oocytes [71, 86, 90, 134]. Using α_{1C}, we have shown that β_3 affects the currents as observed with other βs; it increased currents (> 10-fold), shifted the activation limb of the I-V curve −7 mV, and accelerated the kinetics of activation and inactivation [134]. In addition to altering the inactivation kinetics, β_3 decreased the plateau current attained during long (>3 second) pulses. These results can be interpreted as β_3 altering the equilibrium between open and inactive states. Although the other βs had modest effects on inactivation, it is clear that β_3 has a more profound effect. Studies using α_{1A} reached a similar conclusion [90].

Molecular biology of β_4

Cloning of β_4

β_4 was cloned using PCR with primers that matched conserved regions in β_1 and β_2 [136]. We then isolated a full-length cDNA by screening a rat brain cDNA library. This cDNA contains an open reading frame that encodes a 58 kD protein. Its sequence also contains regions that are highly conserved in all the βs, while the carboxyl terminus is more divergent. A phylogenetic tree based on sequence similarities indicates that β_4 is most closely related to the ancestral β. Alignment of the predicted helices with the hydropathy values shows that none of the helices are hydrophobic [136]. β_4 does not contain any consensus sequences for PKA phosphorylation.

Northern analysis shows that β_4 is predominantly expressed in brain, especially cerebellum [136]. Weaker expression was also detected in kidney. The cross-reactive mRNAs were large (7 kb) appearing as a doublet. Although this may suggest splice variation in the coding region, none has been detected. A likely explanation is that there is alternate use of a polyadenylation signal, as we have found in β_2 [132]. The only alternate splicing detected so far is the deletion of the same small exon found in other βs, which again leads to a frame shift and truncation (β_{4tr}).

Functional expression

β_4 modulates α_{1C} activity the same way as β_3: it stimulates the amount of currents measured tenfold, shifts the IV −10 mV, accelerates activation kinetics, and changes the rate and extent of inactivation [136]. We have also tested the effect of β_4 on endogenous oocyte channels. These channels are stimulated threefold by injection of β_4. However, no effect was observed on either the relative position of the IV curve, or on the activation kinetics (which were quite fast already, 1 to 2 ms) [136]. A small effect on inactivation was measured, but this effect was overlooked until we analyzed single channel recordings, where β_4 was found to decrease the decay of T-like channels [69].

Mechanisms of $\alpha_1\beta$ interaction

In general, βs increase α_1 activity, alter its voltage-sensitivity, and its kinetics. Usually βs accelerate activation and inactivation kinetics. However, β interactions with other αs can have other, sometimes unpredictable, results. For example, β_2 accelerates inactivation of α_{1C} [124], but has the opposite effect on α_{1A} [86], α_{1E} [87], and the endogenous oocyte T- and N-type channels [69]. At the single channel level we found that β_2 affected the endogenous oocyte channels by causing reopenings of the channel. Another notable finding was that β_2 did not affect the peak P_o, which was ~50%. However, βs do increase the peak whole cell current. This leads to the conclusion that βs must increase the number of functional channels on the cell surface. Our previous studies with β increasing the number of DHP binding sites also suggested that βs increase the number of functional channels [37, 132, 136]. Immunoblot studies have suggested that β does not increase the total amount of α_1 protein synthesized [77, 137], however, these studies do not address the key issue, which is, does β increase the amount of α_1 protein expressed at the cell surface.

Studies of gating charge movement in oocytes have reached a different conclusion [138]. Coexpression of β_2 did not increase the amount of charge movement attributed to α_{1C}, although it did increase the inward current. Based on the assumption that the measured gating current reflects the number of expressed channels, Neely et al proposed that βs facilitate the conformational changes that occur between voltage sensing and channel opening. Resolution of this problem requires single channel measurement of P_o. However, this is very difficult to measure because α_{1C} has such a low (< 0.05) and variable P_o when expressed in oocytes. Despite a low P_o that makes it hard to count the number of channels in a patch, Wakamori et al concluded that β increased P_o from 0.012 to 0.041 [66].

These discrepancies may be reconciled if a fraction of α_1 molecules are folded incorrectly, able to sense and move to membrane voltage gradients, but unable to bind DHPs with high affinity. β subunits may act as a chaperonin, helping form Ca^{2+} channel complexes that bind DHPs and flux Ca^{2+}. In fact, Mitterdorfer et al [137] have shown that β does increase DHP binding affinity to α_{1C}, which may have been due to this conversion from low, unmeasurable binding to the typical high affinity binding.

While the mechanisms by which βs modulate the activity of different α_1s may vary, apparently the interaction site is well conserved. This region was discovered by screening an expression library made from α_{1S} cDNA with ^{35}S-labeled β_{1b} protein [139]. This screening isolated many clones that encoded the domain I to II loop. This β-binding domain was mapped in more detail using site-directed mutagenesis. Little information is known about the function of this loop, so it remains a mystery how β modulates α_1 activity.

The site on β that interacts with α_1 has also been mapped [140]. In these studies the β_{1b} subunit was truncated into progressively smaller pieces, then assayed for their ability to stimulate α_{1A}-induced (plus α_2) currents in oocytes. The smallest fragment that stimulated currents contained residues 211 to 245. Incredibly, this region is nearly the same as the "common" exon (residues 217 to 243) [130], which is the first common exon found after the central splice region (shown in Fig. 3). This exon is occasionally skipped, leading to frame shifts and truncation. Since these truncated forms lack this α interaction domain, they are not expected to couple to α_1. Additionally the amino terminus of β subunits is capable of interacting with α_1, and this interaction controls the effect of βs on kinetics [87].

Structure-function of α_1

The significant amount of amino acid sequence identity between Na^+ and Ca^{2+} channels suggests that they have similar structures. Each channel has four domains that each contains eight membrane spanning regions. K^+ channels have a similar predicted structure, but only one domain; four subunits come together to form the channel. This similarity in structure predicts similarities in function. For example, the S4 segment of K^+ and Na^+ channels has been shown to play a major role in the voltage sensor. Although it hasn't been proven yet, it is likely that the S4 segments in Ca^{2+} channels play a similar role.

Other regions of similarity include the pore lining domains. These regions are highly conserved between the Ca^{2+} channel subtypes. Mutations in this region alter ion selectivity and conductance [141–143]. These studies suggest that the selectivity filter is formed by a spiral staircase of glutamates contributed by each domain [142].

A very useful strategy in elucidating structure-function relationships has been to make chimeras between the different α_1 subtypes. Tanabe and coworkers [144] were the first to apply this strategy to calcium channels, and by making chimeras between α_{1S} and α_{1C} they showed that the Domain II-III loop is required for skeletal muscle excitation-contraction coupling. They have also shown that Domain I, in particular S3 and S3-S4 linker, contains regions involved in determining activation kinetics [145, 146]. Using similar approaches the Domain I S6 region was shown to be involved in voltage-dependent inactivation [147], while the putative E-F hand located after Domain IV was implicated in calcium-dependent inactivation [148].

Dihydropyridine binding regions have also been identified using biochemical and molecular biological techniques [149–151]. By making chimeras between the DHP-sensitive α_{1C} and the insensitive α_{1A}, Tang et al showed that one of the DHP interaction sites is near the domain IV pore region [151]. A complete description of the DHP binding pocket may provide insights into the three-dimensional structure of the channel.

Summary

Pharmacological and electrophysiological studies have established that there are multiple types of voltage-gated Ca^{2+} channels. Molecular biology has uncovered an even greater number of channel molecules. Thus, the molecular diversity of Ca^{2+} channels has its basis in the expression of many α_1 and β genes, and also in the splice variants produced from these genes. This ability to mix and match subunits provides the cell with yet another mechanism to control the influx of calcium. Future studies will describe new subunits, the subunit composition of each type of channel, and the cloning of new Ca^{2+} channel types.

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